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APPLICANTS: Peter J. Quesenberry

EXAMINER: V. Afremova

SERIAL NO. 10/562,086

GROUP: 1657

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FOR: METHODS OF PRODUCING DIFFERENTIATED HEMATOPOIETIC
CELLS FOR TREATMENT OF CYTOPENIA

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Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. PETER J. QUESENBERY PURSUANT TO 37 CFR 1.132

I, Dr. Peter J. Quesenberry, declare as follows:

1. I am presently a Professor of Medicine at the Warren Alpert Medical School of Brown University and Rhode Island Hospital in Providence, RI (since 2006); a Professor of Medicine at Boston University in Boston, MA (since January 2001); Director of the Division of Hematology/Oncology at Brown Medical School and Lifespan Medical Center at Rhode Island Hospital and Miriam Hospital in Providence, RI (since October 2006); the Paul Calbresi, MD, Professor in Oncology at Brown University and Rhode Island Hospital in Providence, RI (since October 2006); a member of the Board of Trustees of Roger Williams Medical Center in Providence, RI (since 2004); and a member of the National Board of Trustees for the Leukemia Society of America (since 1984). I hold active licenses to practice medicine in Virginia (1964-present), Massachusetts (1993-present), and Rhode Island (2001-present). I am a Diplomate in the specialty of International Medicine (1971) and in the

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subspecialties of Hematology (1972) and Medical Oncology (1975). I currently serve on the Cancer Committee (since 2006), the SOP Committee (since 2006), the Oncology Council (since 2006), the Curriculum Committee (since 2007), the Promotions Committee (since 2006), the Cancer Control Committee (since 2006), the Joint Protocol Committee (since 2006), and the Clinical Practice Group (since 2006) at Rhode Island Hospital. In addition, I am the Vice Chairman of the Board of the Leukemia/Lymphoma Society of America (since 2000); a member of the NASA Grant Review Committee (since 1998); Chair of the Translational Research Grant Review Committee, LLSA (since 1995); and a member of the American Society for Blood and Marrow Transplantation (since 1994). I have also served as the Chair of the Department of Research (2001-2006), a member of the Bylaws Committee (2002-2006), the Head of the Resource Allocation Committee (2002-2006), and the Director of the Adele R. DeCof Cancer Center and the Blood and Marrow Transplant Program (2004-2006), all at Roger Williams Medical Center in Providence, RI. During this time, I have served on various committees at Roger Williams Medical Center and Rhode Island Hospital (2001-present). I was also a Professor of Medicine (1993-2000) and a Professor of Cell Biology (1993-2000) at the University of Massachusetts Medical Center and in the Graduate School of Biomedical Science in Worcester, MA, where I also served as Interim Chief of Radiation Oncology (1993) and Director of the Cancer Center (1993-2000). During this time I served as a member of various committees at the University of Massachusetts in Worcester, MA (1993-2000). Prior to that time, I was a Professor of Medicine (1979-1992), the Byrd S. Leavell Professor of Medicine (1981-1992), a Professor of Otolaryngology (1981-1992), Chief of the Division of Hematology/Oncology (1979-1992), Director of the Inpatient Hematology/Oncology Unit (1984-1992), Associate Director for Research and Education of the Cancer Center (1984-1991) at the University of Virginia School of Medicine in Charlottesville, VA. During this time I served as a member of various committees at the University of Virginia School of Medicine and was Chief of the Hematology/Oncology Clinic at the Primary Care Center at the University of Virginia and Chair of the Institutional Research Grant Committee of the American Cancer Society (1979-1983). I have also held positions as an Assistant Professor of Medicine at Harvard Medical School in Boston, MA

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(1976-1979) and as Assistant Professor of Medicine (1973-1976) and Instructor in Medicine (1972-1973) at Tufts Medical School in Boston, MA. I have also served as Vice President (2001-2002), President-Elect (2002-2003), and President (2003-2004) of the International Society for Experimental Hematology in Washington, DC; as Vice President of the Leukemia Society of America, Medical and Scientific Affairs (1989-1992); on the Executive Committee of the American Society of Hematology (1985-1988 and 1992-1995); on the Board of Directors of Hospice of Central Massachusetts Visiting Nurses Association (1995-1996); as President of the Southern Society of Clinical Investigation Blood Club, Southern Section (1984-1985); on the Board of Directors of the American Blood Commission (1983-1984); on the MKSAP Hematology Subcommittee of the American College of Physicians (1983); on the American Board of Internal Medicine Task Force on Hematology and Medical Oncology (1983); as President of the Virginia Society of Hematology (1982-1983); and on the Board of Trustees of the Leukemia Society of America, Virginia Chapter (1980-1982). I have also served on the American Society of Hematology Publications Committee (1997), Growth Factor Education Session (1988, 1989, 1991), Erythropoietin and Cell Proliferation Subcommittee (1988-1989), and Scientific Affairs Committee (1978-1979) and as Chair of the Training Grant Committee (1991), the Growth Factor Subcommittee (1988-1989), and the Leukocyte Physiology Subcommittee (1981); on the Patient Aid Committee (1995-1998); on the National Heart, Lung, and Blood Institute Program Project Review Committee (1994-1998); on the National Bone Marrow Donor Registry Advisory Committee (1993-1994); on the American Cancer Society Scientific Advisory Committee on Chemotherapy and Hematology (1992-1993); as a member of the Association of Hematology and Oncology Program Directors (1991-1994); on the U.S. National Committee for the International Union Against Cancer (1990-1994); as Vice President of Medical and Scientific Affairs, LLSA (1989-1992) and as Chair of the Professional Education Committee, LLSA (1986-1988); on the Ortho Pharmaceuticals Advisory Board (1988-1992); on the NHLBI Blood Diseases and Resources Advisory Committee (1988-1991); on the National Institutes of Health (NIH) Hematology Study Section (1988-1991), as Chair of the NIH Ad Hoc AIDS Study Section (1988), on the NIH Hematology Study Section (1980-1984), and on the NIH Erythropoietin

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Distribution Subcommittee (1979); as a member of the Standard Grant Review Committee for the Leukemia Society of America (1986-1994); as a Chairman (1985) and on the Visiting Committee (1982-1986) at Brookhaven National Laboratory; as a Fellow of the American College of Physicians (1983); on the Committee on Hematology of the American Board of Internal Medicine (1982-1986); on the ISEH Membership Committee (1980-1982); on the National Institute on Arthritis and Metabolic Diseases Subcommittee on Normal Hemopoiesis (1979-1980); on the Frederick Stohlman, Jr., MD, Memorial Symposium on the Etiology and treatment of Leukemia (1974-1979); and as a Scholar with the Leukemia Society of America (1972-1977). I have authored or co-authored at least 200 original publications in peer-reviewed journals, as well as more than 70 other peer-reviewed publications, more than 90 books or book chapters, and more than 340 abstracts. I have received numerous grants, and since 1993, I have given over 195 invited presentations. I hold a Bachelor of Arts (1960) from the University of Virginia in Charlottesville, VA, where I majored in English and was a member of the Raven Society and in the IMP Society (1959). I hold a Doctor of Medicine (M.D.) (1964) from the University of Virginia in Charlottesville, VA, where I was a member of the honor society Alpha Omega Alpha (1964). I was a resident at Boston City Hospital (Boston University Service) in Boston, MA (1967-1969); a fellow in Clinical Hematology at St. Elizabeth's Hospital in Brighton, MA (1969-1970); and a fellow in Research Hematology at St. Elizabeth's Hospital in Brighton, MA (1970-1972). I attained the rank of Lieutenant as a medical officer in the United States Navy (1965-1967). I am a member of a number of professional societies, have served on numerous professional committees and advisory boards, and have been the recipient of various honors and awards, including the Kenny Award (1992), the National Leadership Award (1998), the Merit Award (1999), and the 50th Anniversary Commemorative Award (1999) from the Leukemia Society of America and the Lifetime Achievement Award (2006) from the Leukemia/Lymphoma Society of America.

2. The subject application discloses among other things and claims a method for the production of cell cycle specific differentiated hematopoietic cells comprising: a) culturing purified bone marrow stem cells for cycle initiation from resting state under

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conditions that promote synchronous progression through the cell cycle; b) selecting at least one desired cell cycle specific hematopoietic cell type to be differentiated from the synchronous purified bone marrow stem cells upon contact with at least one growth factor or cytokine at a phase of the cell cycle favoring a specific differentiation pathway for the at least one desired cell cycle specific hematopoietic cell type; c) determining the phase of the cell cycle favoring the specific differentiation pathway for the at least one desired cell cycle specific hematopoietic cell type, and selecting the at least one growth factor or cytokine to promote the specific differentiation pathway or to produce a plurality of cells of the at least one desired cell cycle specific hematopoietic cell type to be differentiated from the synchronous purified bone marrow stem cells upon with at least one growth factor or cytokine at a phase of the cell cycle with the at least one growth factor or cytokine at the predetermined phase of the cell cycle; d) contacting the synchronous purified bone marrow stem cells with the at least one growth factor or cytokine at the predetermined phase of the cell cycle; and e) subculturing the cells until cell cycle specific differentiated hematopoietic cells are produced, wherein a plurality of the cell cycle specific differentiated hematopoietic cells have the at least one desired cell cycle specific hematopoietic cell type. It also discloses among other things and claims a method for the production of cell cycle specific differentiated hematopoietic cells comprising: a) culturing purified bone marrow stem cells for cycle initiation from resting state under conditions that promote synchronous progression through the cell cycle; b) contacting the cells with at least one growth factor or cytokine at a predetermined phase of the cell cycle; and c) subculturing the cells until cell cycle specific differentiated hematopoietic cells are produced, wherein: i) the predetermined phase of the cell cycle is mid-S phase and the differentiated hematopoietic cells comprise megakaryocytes, platelets, or proliferative granulocytes; or ii) the predetermined phase of the cell cycle is late S phase and the differentiated hematopoietic cells comprise mature (non-proliferative) granulocytes.

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3. In general, models of stem cell regulation have been hierarchical. In these models, a primitive stem cell having great potential give rise to a proliferating progenitor pool, which in turn gives rise to recognizable differentiated cells. During this process, proliferative potential is lost, while specific differentiated features are acquired. Presumptively, but without definite proof, there is also self-renewal at the most primitive stem cell level, and this self-renewal is also lost with differentiation. Many data exist to support such a hierarchical model. Marrow cells have been separated with short-and long-term repopulation potential, and progenitors have been characterized as exclusively committed to the production of restricted progeny. In addition, the clear expansion of different progenitor types in cytokine-stimulated in vitro culture with a loss of long-term engraftment capacity speaks to the existence of a progenitor hierarchy, at least at the more differentiated levels. Not all data fit this model, however. It has been observed, for instance, that "daughter cell" or paired-progenitor experiments indicate that a primitive progenitor spleen cell can make totally different lineage choices during one cell cycle transit, such that, for example, one daughter cell forms one type of cell colony, while a different daughter cell forms a different type of cell colony. An intrinsic component of the hierarchical model is that the most primitive hematopoietic stem cell is a quiescent cell in G_0 having a fount of potential without differentiated characteristics. It has generally been believed that primitive hematopoietic stem cells were dormant or quiescent and were thus protected from depletion or exhaustion. On the basis of a number of transplant studies, a clonal succession model has been proposed. This model proposed that the production of blood cells is maintained sequentially by one or just a few lymphohematopoietic stem cells, the residual stem cells remaining dormant. This model forms the basis for the concept that hematopoietic stem cells are hierarchically ordered based on their relative quiescence. In chemotherapy and/or stem cell transplantation approaches, a major risk remains the cytopenic period occurring before stem cells have engrafted and produced differentiated progeny. The administration of more differentiated progenitor cells which might rapidly produce desired cell populations has intrigued investigators, but has been limited by the number of available progenitor cells for such transplant support.

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4. The current invention addresses these concerns and many other issues as well.

5. I have reviewed the Patent Office Action ("Office Action") dated January 23, 2009, issued in connection with the subject application. As I understand the Office Action, the Patent Examiner has rejected certain claims of the application in view of documents that include the following: Hagihara et al. (J. Immunol. Methods, 2001, 253: 45-55; "Hagihara"); Feng Yan et al. (Blood, November 2000, 96(11)(part 1): 680a; "Feng Yan" or "Yan"); Klabusay et al. (Blood, November 2002, 100(11): Abstract 4118; "Klabusay"); Ramsfjell et al. (Blood, December 1996, 88(12): 4481-4492; "Ramsfjell"); and Messner et al. (Blood, November 1987, 70(5): 1425-1432; "Messner").

6. I disagree with these claim rejections.

7. The current invention illustrates that it is possible to produce differentiated hematopoietic cells (e.g., megakaryocytes, platelets, and/or granulocytes) *in vitro* by culturing synchronized bone marrow stem cells in the presence of one or more cytokines (e.g., G-CSF, GM-CSF, and/or steel factor) at a specific, predetermined phase of the cell cycle (e.g., mid-S phase or late S phase) and subculturing the cells until differentiated hematopoietic cells are produced. Specifically, the discoveries of the instant invention establish the existence of differentiation "hotspots" at certain times in the cell cycle, that is, points where a specific differentiation pathway is favored.

8. In contrast to the present application, Hagihara is directed to a method of ex vivo production of dendritic cells from CD34 positive umbilical cord blood or bone marrow cells using mouse stromal cells and a HESS-5 culture system with three cytokines (Flk-2/Flt-3 ligand, stem cell factor, and thrombopoietin) to produce primitive myeloid cells which can develop into dendritic cells. Unexpectedly, we have found that bone marrow stem cells continually change phenotype in a reversible fashion tied to cell cycle transit (the "continuum theory"). The present application addresses a portion of this concept, namely, the ability to

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cause cells to differentiate into various cell types linked to one phase of the cell cycle vs. another phase of the cell cycle, which provides the capacity to predict a differentiation cascade based on precise synchronization of the stem cell cycle in the presence of an appropriate inducing stimulus. However, as even the Office Action admits, Hagihara fails to disclose or suggest that the cell culturing in the presence of steel factor (SCF), thrombopoietin (TPO) and FLT-3 ligand (FLT3) promotes synchronous progression of cells through the cell cycle. In addition, not only does Hagihara fail to disclose synchronous progression of cells through the cell cycle, Hagihara also does not disclose contacting synchronously cycling cells with a growth factor or cytokine "at a predetermined phase of the cell cycle." Instead, the cells in Hagihara were subsequently subcultured "every week" with no disclosure regarding the timing of the subculturing with regard to the cell cycle. Hagihara does not even disclose whether the subculturing of the cells took place at the same time of day on the same day of "every week." the claims are limited by the phase of the cell cycle, which Hagihara is not. Rather, Hagihara discloses a vague, imprecise, and random period of time ("every week" – without even any discussion of the day, time, or duration of cell cycle), unlike the Applicants, whose claims are directed to a specific phase of the cell cycle ("predetermined phase of the cycle"), regardless of when in time that phase occurs. In essence, Applicants are claiming a method based on cell cycle, while Hagihara discloses a method based on time. Even if it were possible to know the duration of the cell cycle in numbers of hours (and it is not), Hagihara's vague, imprecise, and random "every week" neither discloses nor suggests a consistently specific cell cycle phase. Therefore, that it cannot be concluded that Hagihara subcultured synchronously cycled cell cultures with a growth factor or cytokine "at a predetermined phase of the cell cycle." Additionally, Hagihara neither describes nor suggests the selection of a desired cell cycle specific hematopoietic cell type to be differentiated from the synchronous purified bone marrow stem cells at a phase of the cell cycle favoring a specific differentiation pathway for the desired cell cycle specific hematopoietic cell type followed by the selection of at least one growth factor or cytokine to promote the specific differentiation pathway or to produce a plurality of cells, having the desired cell cycle specific hematopoietic cell type, from the synchronous purified

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bone marrow stem cells when the synchronous purified bone marrow stem cells are contacted with the growth factor or cytokine at the predetermined phase of the cell cycle. There is no disclosure in Hagihara of the selection of these factors with respect to the "predetermined phase of the cell cycle" with respect to favoring a specific differentiation pathway or generating a plurality of cells having the desired cell cycle specific hematopoietic cell type when the synchronous purified bone marrow stem cells are contacted with the growth factor or cytokine at the predetermined phase of the cell cycle.

9. Hagihara, alone or in combination with the other references, fails to teach or suggest the present invention.

10. In contrast to the present invention, Yan is directed to a combination of cytokines (thrombopoietin, Flt-3 ligand, stem cell factor and IL-6) used to induce cell cycling and proliferation of a stem cell population with development of a differentiated phenotype during 7 days in culture. With respect to Yan, the Yan culture was quiescent (97.4% G₀/G₁ phase; 1.5% S phase), but was stimulated by cytokines to enter into cycle as early as 24 hours to yield a fast-dividing population and a slow-dividing population, whereas the cells of the present invention are cultured from dormancy to synchronous cycles and then stimulated by exposure "at a predetermined phase of the cell cycle," namely, a two-step process – first synchronization, then differentiation into the desired cell cycle specific type.

11. Yan, either alone or in combination with Hagihara and/or the other references, fails to teach or suggest the unexpected ability to cause cells to differentiate into various cell types linked to one phase of the cell cycle vs. another phase of the cell cycle, in essence, the capacity to predict a differentiation cascade based on precise synchronization of the stem cell cycle in the presence of an appropriate inducing stimulus, as in the present invention.

12. In contrast to the present invention, Klabusay is directed to showing that hematopoietic stem cells are able to regenerate hematopoiesis in all lineages and that addition

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of G-CSF in particular will significantly increase the number of matured cells including granulocytes. Klabusay neither discloses nor suggests generation of at least one specific hematopoietic cell type from a synchronous population of stem cells by exposure to at least one growth factor or cytokine "at a predetermined phase of the cell cycle."

13. Klabusay, either alone or in combination with Hagihara, Yan, and/or the other references, fails to teach or suggest the unexpected ability to cause cells to differentiate into various cell types linked to one phase of the cell cycle vs. another phase of the cell cycle, in essence, the capacity to predict a differentiation cascade based on precise synchronization of the stem cell cycle in the presence of an appropriate inducing stimulus, as in the present invention.

14. In contrast to the present invention, Ramsfjell is directed to showing that the use of factor SCF enhances megakaryocyte differentiation and production from stem cells. Ramsfjell neither discloses nor suggests generation of at least one specific hematopoietic cell type from a synchronous population of stem cells by exposure to at least one growth factor or cytokine "at a predetermined phase of the cell cycle."

15. Ramsfjell, either alone or in combination with Hagihara, Yan, Klabusay, and/or the other references, fails to teach or suggest the unexpected ability to cause cells to differentiate into various cell types linked to one phase of the cell cycle vs. another phase of the cell cycle, in essence, the capacity to predict a differentiation cascade based on precise synchronization of the stem cell cycle in the presence of an appropriate inducing stimulus, as in the present invention.

14. In contrast to the present invention, Messner is directed to showing that cell cycle studies and stem cell engraftment studies indicate that higher than normal proportions of multipotential hematopoietic cells are present in S phase during progression of the hematopoietic cells through the cell cycles. Messner does not distinguish between mid-S

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phase and late S phase with respect to generation of different differentiated hematopoietic cell types. Messner neither discloses nor suggests generation of at least one specific hematopoietic cell type from a synchronous population of stem cells by exposure to at least one growth factor or cytokine "at a predetermined phase of the cell cycle."

17. Messner, either alone or in combination with Hagihara, Yan, Klabusay, and/or Ramsfjell, fails to teach or suggest the unexpected ability to cause cells to differentiate into various cell types linked to one phase of the cell cycle vs. another phase of the cell cycle, in essence, the capacity to predict a differentiation cascade based on precise synchronization of the stem cell cycle in the presence of an appropriate inducing stimulus, as in the present invention.

18. In the present application, the data showed that Lineage^{negative}Rhodamine^{low}Hoescht^{low} ("LRH") cells synchronized and then sub-cultured separately inductive differentiation cocktail (GM-CSF, G-CSF, and steel factor) prior to cell division showed marked variations in differentiated cell production with the first cell cycle transit (Example 1; Figures 1-4). Surprisingly, megakaryocyte differentiation and proliferative granulocyte differentiation were amplified at G₀ to mid-S phase, whereas non-proliferative granulocyte differentiation was amplified at G₀ to late S phase (Example 1; Figures 1-6).

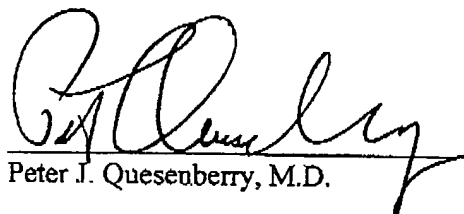
19. The results are surprising, because these differences provide evidence for a flexible system for hematopoietic regulation in which multiple different outcomes can occur dependent on cell cycle phase and specific microenvironmental influences as part of a continuum of reversible phenotypic shifts (in contrast to a hierarchical model) with continuous change in a reversible fashion.

20. None of the references cited by the Patent Office addresses this concept or teaches or suggests this concept.

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21. I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 6/11/09


Peter J. Quesenberry, M.D.